Recombinant integrin CD11b A-domain blocks polymorphonuclear cells recruitment and protects against skeletal muscle inflammatory injury in the rat

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Summary

The β2 integrin CD11b/CD18 (CR3) is a major adhesion receptor of neutrophils, normally utilized to fend off infections. This receptor contributes, however, to multiple forms of non-infectious inflammatory injury when dysregulated as shown in gene knock-outs and through the use of blocking monoclonal antibodies. The major ligand recognition site of CR3 has been mapped to the A-domain in the CD11b subunit (CD11bA). The recombinant form of this domain exhibits a ligand binding profile similar to that of the holoreceptor. To assess the potential anti-inflammatory activity of CD11bA as a competitive antagonist of CR3 *in vivo*, we assessed its effects on a developed animal model of traumatic skeletal muscle injury in the rat. Recombinant soluble rat CD11bA-domain fused to glutathione-S-transferase (GST) was administered intravenously in a single dose at 1 mg/kg to nine groups of Wistar rats, five in each group, 30 min before inducing traumatic skeletal muscle injury. Control animals received either a function-blocking anti-CD11b/CD18 monoclonal antibody (1 mg/kg), non-functional mutant forms of the CD11bA (D140GS/AGA, T209/A, D242/A), recombinant GST or buffer alone. In control animals, the wounded muscle showed oedema, erythrocyte extravasation and myonecrosis both within and outside the immediate wounded area (5–10 mm zone) and influx of neutrophils was detected 30 min post-wound, followed by a second wave 3 hr later. Wild-type CD11bA- or anti-CD11b monoclonal antibody (mAb)-treated rats showed a comparable and significant decrease in the number of infiltrating PMN (78 + 4%, $n = 70$ and 86 ± 2%, $n = 50$, respectively) and preservation of the muscular fibres outside the immediate zone of necrosis (75 + 4%, $n = 70$, 84 ± 1%, $n = 50$, respectively), compared to controls. These data demonstrate that CD11bA can be an effective tissue-preserving agent in acute inflammatory muscular injury.

Keywords: adhesion molecules; inflammation; integrins; ischaemia-reperfusion; muscle trauma

Introduction

In response to infectious or noxious agents, circulating neutrophils (PMN) guided by locally released chemokines are recruited into tissues,[1] where they participate in immune clearance, a vital component of host response against infection. In many non-infectious inflammatory diseases, however, neutrophils are activated inadvertently and recruited to tissues where they participate in further damage[2] through release of oxygen free radicals and

Abbreviations: ANOVA, analysis of variance; CR3, complement receptor 3; GST, glutathione-S-transferase; ICAM1, intercellular adhesion molecule 1; mAb, monoclonal antibody; MIDAS, metal-ion-dependent-adhesion-site; NOD, non-obese diabetic; PMN, polymorphonuclear cells; sr, soluble recombinant; vWF, Von Willebrand factor.

proteolytic enzymes, thus compromising the function of vital organs. CR3 (CD11b/CD18) is the major β2 integrin expressed in neutrophils. It is utilized in the recruitment of these cells to inflamed tissues[3,4] and has therefore been an important therapeutic drug target for tissue preservation in harmful inflammatory states.[5,6]

The CD11b/CD18 heterodimer is a versatile adhesion molecule that plays a key role in the firm attachment of circulating leucocytes, primarily neutrophils, to the vascular endothelium as well as in transendothelial migration into tissue, which are markedly enhanced in inflammatory diseases.[7,8] The extent of this infiltration determines the outcome of numerous infectious or inflammatory conditions. Indeed, many diseases result from or are exacerbated by unregulated extravasation of these inflammatory cells. In these instances, infiltrating inflammatory cells cause bystander damage to an already injured organ such as the heart (following a myocardial infarction),[9,10] lung,[11,12] joints (rheumatoid arthritis),[13,14] skin (burns),[15,16] allograft (immune injury),[17–19] gut (ulcerative or ischaemic colitis)[20] and nervous system (multiple sclerosis).[21] This damage can compromise the function of these organs, and cause morbidity and mortality. In experimental animal models of inflammation, anti-CD11b or anti-CD18 monoclonal antibodies (mAbs) efficiently blocked neutrophil extravasation. Myocardial reperfusion injury was significantly reduced following the use of anti-CD11b and/or anti-CD18 mAbs in dogs and primates.[22–24] In the non-obese diabetic (NOD) mouse, an established animal model of human juvenile diabetes mellitus, mAbs to CD11b prevented the infiltration of islet cells by both macrophages and T cells and inhibited the development of diabetes.[25]

The proinflammatory effects mediated by CR3 are initiated upon ligation of the integrin by one or more ligands. CR3–ligand interaction requires that the receptor switches from a low- to a high-affinity state, competent in ligand binding.[26] The ligand binding function of CR3 has been mapped to a ~190 amino acid domain located in the NH2 terminus of the CD11b-subunit. This Von Willebrand factor (vWF) A-type domain (named A- or I-domain) is also present in nine other integrin subunits (for review see Xiong *et al.*[27]). The crystal structure of the CD11b A-domain (CD11bA) revealed a metal-ion-dependent-adhesion-site (MIDAS),[28] which coordinates a metal ion that is necessary for the ligand binding function of the domain as well as the native heterodimer. The MIDAS motif comprises five non-contiguous amino acids, namely D140, S142, S144, T209 and D242. Mutations in any one of these residues abolish metal ion and ligand binding.[29] Firm adhesion of PMN to vascular endothelium is mediated by the binding of CR3 to ligands expressed on the endothelial cell surface, including intercellular adhesion molecule 1 (ICAM1) (CD54).[30] Recombinant soluble forms of human and rat CD11bA have been generated and shown to bind to CR3 physiological ligands such as iC3b, ICAM1 and fibrinogen *in vitro*.[29,31–33] Soluble forms of P-selectin, an endothelial cell adhesion, blocked neutrophil adhesion to endothelium and prevented neutrophil accumulation into inflammatory sites.[34,35] These observations led us to investigate the possibility of controlling leucocyte influx into inflamed skeletal muscle *in vivo*, using a soluble recombinant (sr) form of CD11bA.

In this paper, we tested the ability of recombinant CD11bA to act as an anti-inflammatory agent *in vivo* in a rat model of mechanically induced skeletal muscle injury we have developed. Purified rat srCD11bA–glutathione-S-transferase (GST) fusion protein administered intravenously prior to mechanical injury blocked leucocyte infiltration and effectively prevented muscle damage. Non-functional forms of srCD11bA were ineffective. srCD11bA is therefore a useful tissue preserving-agent in traumatic muscle injury and potentially in other acute inflammatory states.

Materials and methods

Reagents and mAbs

Restriction and modification enzymes, gluthatione sepharose and Mono-Q columns and the pGEX-2T bacterial expression vector were purchased from Amersham-Pharmacia Biotech, Uppsala, Sweden. Murine mAb to rat CD11b/c, OX42 (IgG2A) and the isotype-matched negative control mAb G155-178 were purchased from Pharmingen (San Jose, CA). The anti-GST mAb G172-1138 was from Amersham-Pharmacia Biotech.

Animals

Inbred Wistar female rats weighing 200–220 g were used to develop the muscle injury model, in accordance with institutional guidelines and in conformity with the international standards recommended for animal experimentation. One hundred and fifteen groups of five rats each were used for this study (72 groups for developing the model and 39 to assess the anti-inflammatory potential of srCD11bA). Four animal groups did not undergo muscle injury and were used as untreated controls.

Construction of wild-type and mutant rat srCD11b-encoding cDNAs

The rat CD11bA coding sequence was amplified using the *Pycococcus furiosus* (PFU) DNA polymerase, from a rat CD11b cDNA (Zerria & Fathallah, GenBank Accession no. AF268593) and inserted into the pGEX2T expression vector downstream of the GST coding sequence in two subcloning steps: first a 150 base pairs (bp) DNA

fragment, prepared by a *Bam*HI/*Eco*RI digestion of the amplified CD11bA and corresponding to the 5′ end of rat CD11b A was ligated using T4 ligase to the corresponding sites of the vector polylinker. This construct was digested with *Eco*RI and ligated to a 450 bp *Eco*RI DNA fragment corresponding to the rest of the CD11b A.

Polymerase chain reaction (PCR) site-directed mutagenesis[36] was performed in pGEX-2T CD11bA vector using phosphorylated mutagenic primers designed to have mismatches introduced at their 5′ ends. Primers agaF: 5′-CCGGCGCCGGTAGCATCAACA-3′ and agar: 5′-AA TCAAGAAGGCAATGTTGCT-3′ (for the D140GS/AGA mutation); t209aF: 5′-GGTGCCTCAGGGATCCGTAA-3′ and t209aR: 5′-TTTTGTCCTTCCATTCAGCT-3′ (for the T209/G mutation) and d242aF: 5′-ccggagaaaagtttggtgac-3′ and d242aR: 5′-CTGTGATGACAACTAGGATC-3′ (for the D242/A mutation) were used. The primers were annealed to plasmid pGEX-2T rat CD11bA and the PFU DNA polymerase (Stratagene, Amsterdam, the Netherlands) was used to perform 30 PCR amplification cycles at the following conditions: 94° for 45 seconds, 55° for 1 min and 72° for 2·5 min followed by 5 min at 72°. The blunt-ended PCR fragment was gel purified, ligated using an excess of T4 DNA ligase and used to transform *Escherichia coli* DH5 competent cells. Wild-type and mutant pGEX-2T rat CD11bA clones were fully checked by nucleotide sequencing using the Applied Biosystem ABI Prism 377 DNA Sequencer before being used to produce the srCD11bA–GST fusion proteins.

Production and purification of rat srCD11bA fusion peptides

Wild-type and mutant rat srCD11bA–GST fusion proteins were produced as described by Mischishita *et al.*,[29] except that the *E. coli* BL21 strain was used and cells were harvested 8 hr after induction with isopropyl thiogalactose (IPTG) (0·1 mM). Purification of the fusion proteins was carried out on a gluthatione sepharose column, followed by fast protein liquid chromatography (FPLC) using a Mono-Q column. Purity was checked in a 12% sodium dodecyl sulphide (SDS)–polyacrylamide gel stained using Coomassie blue, and the fusion protein was visualized by chemiluminescence using anti-GST and/or anti-rat CD11b mAb. Protein concentration was measured using the Bio-Rad protein assay system. The typical protein yield was 10 mg/l of bacterial culture. Recombinant GST was produced using the original pGEX-2T vector following the same procedure.

Rat CD11b A-domain protein modelling

Human CD11b A-domain (PDB id: 1bho)[29] was used as a template sequence. Alignment was performed using ALIGN in MODELER 4.[37] Homology modelling of the rat CD11bA starting from Asn30 to Gly218 was generated using MODELER 4.

Development of the rat model of skeletal muscle injury

Animals were anaesthetized intraperitonially using ketamine and the muscles in both limbs were punctured using a 20-gauge needle mounted on a manual leather-puncturing device to create a haematoma. The rats were killed by intravenous injection of an overdose of the anaesthetic at different time-points varying from 15 min to several days post-injury. The wounded muscles were resected, formalin-fixed and paraffin-embedded; 4–5 μm thick sections were then cut and stained with haematoxylin and eosin for light microscopy examination. Histological observations were performed on a Zeiss axioskop light microscope using an eyepiece graticule grid. Myeloperoxidase (MPO) activity was detected colorimetrically using α-napthol–pyronine (Kit Myeloperoxydase RAL, Reactifs RAL, France) according to the manufacturer's instructions.

Administration of mAbs and srCD11bA peptide

Rats were kept for 20 min at 37° to allow vasodilatation and facilitate injection in the caudal vein. Purified, sterile wild-type srCD11bA–GST fusion protein was administrated intravenously in 200 μl of sterile phosphate-buffered saline (PBS) to nine groups of five rats each, at 1 mg/kg body weight, 30 min before injury. Mutant forms of the fusion protein were each administrated to four groups of rats. Six other control groups of rats received either purified GST (three groups) at 1 mg/kg or 200 μl of sterile PBS alone (three groups). mAb OX42 or mAb G155-178 (isotype control) were administered (1 mg/kg body weight) to five and three groups of five rats, respectively. Four other groups of animals received wild-type srCD11bA–GST at a concentration of 2 mg/kg body weight. Animals were generally killed 3–4 hr post-injury. Two of the nine groups of rats treated with srCD11bA were killed 7–8 hr post-injury. All recombinant peptides and mAb solutions injected had no detectable endotoxin contamination as assessed by the limulus assay (BioWhitaker, Verviers, Belgium).

Assessment of leucocyte transendothelial migration

Leucocytes that transmigrated into the inflamed muscle 5–10 mm away from the traumatic hematoma lesion were counted 3·5 hr post-injury within the graticule grid of 10 microscopic fields. The average number observed in the non-treated group was assigned a value of 100%.

Evaluation of muscle injury

The 10 microscopic fields 5–10 mm away from the traumatic haematoma lesion were also explored for necrotic

muscular lesion. The extent of the necrosis was scored visually as follows: + mild, ++ moderate, +++ severe. The average number of fields in which moderate and/or severe necrosis was observed in the non-treated group was assigned a value of 100%.

Statistics

Data are reported as the mean ± standard deviation (SD). The number of infiltrated leucocytes in the experimental groups was compared using an analysis of variance table (ANOVA) with correction for multiple comparisons when appropriate. Differences were considered significant when $P < 0{\cdot}01$.

Results

Production and purification of recombinant soluble forms of the rat CD11bA peptide

The rat CD11b A-domain coding nucleotide sequence corresponding to residues 125–237 was cloned into the pGEX-2T bacterial expression vector downstream of the GST sequence (Fig. 1a). The purified 45 kDa rat rsCD11bA–GST fusion protein migrated as a single band following SDS–polyacrylamide gel electrophoresis (PAGE) fractionation and Coomassie staining (Fig. 1b), which reacted in Western blots with the function-blocking murine mAb OX42 that recognizes both CD11b/c (Fig. 1c). Alanine substitutions of the MIDAS residues D140, S142, T209, D242 that are involved in metal ion coordination (Fig. 2b) were made in rat CD11bA and the respective mutants were produced with the same yield as the wild-type in bacteria. All three mutants reacted with mAb 0 × 42 in Western blots (data not shown), indicating that none of the mutations affected protein folding.

Development of a rat model of skeletal muscle inflammatory injury

Mechanical injury of skeletal muscle was induced in anaesthesized female Wistar rats by puncturing the muscle in the hind limbs using a 21-gauge needle. The size of the resulting haematoma was reproducible and varied between 7 and 10 mm. An outline of the model, profiles of inflammatory cells recruited and tissue injury are

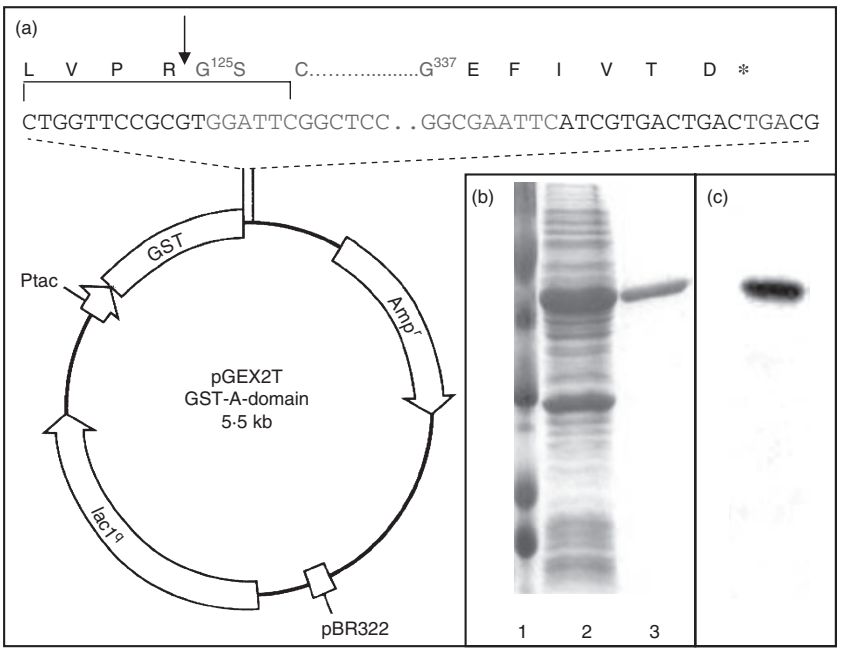

Figure 1. Expression and analysis of rat recombinant CD11bA peptide. (a) Construction of the recombinant A-domain: two *Bam*HI/*Eco*RI and *Eco*RI/*Eco*RI fragments [encoding residues C127 to G337 (magenta) of the native rat CD11b subunit] excised from the full-length CD11b cDNA were subcloned in two steps into the *Bam*HI (in blue) and *Eco*RI (in red) sites of plasmid pGEX2T polylinker. The fusion protein contains six additional residues at the C-terminus followed by a stop codon (*). The thrombin cleavage sequence (LVPRGS) is indicated and the cleavage site is pointed by an arrow. The GS dipeptide is integral to the A-domain which therefore begins with G125 and ends with G337 in the native sequence. (b) Coomassie blue stain of a sodium dodecyl sulphide–polyacrylamide gel electrophoresis (SDS-PAGE) (12·5%) following electrophoresis under reducing conditions: lane 1, prestained molecular size protein markers (200 kDa, 97·4 kDa, 66 kDa, 46 kDa, 30 kDa, 20·1 kDa and 14·3 kDa). Lane 2, isopropyl thiogalactose (IPTG)-induced bacterial lysate harbouring the fusion protein. Lane 3, fast protein liquid chromatography (FPLC) purified rsCD11bA–glutathione-S-transferase (GST) fusion protein at ∼ 45 kDa. (c) Western blot of a gel run under non-reducing conditions and electroblotted onto Immobilon-P membrane, showing the reactivity of the fusion protein with the anti-rat CD11b monoclonal antibody OX42 (Pharmingen). Protein was detected using the enhanced chemiluminescence (ECL) system from Amersham.

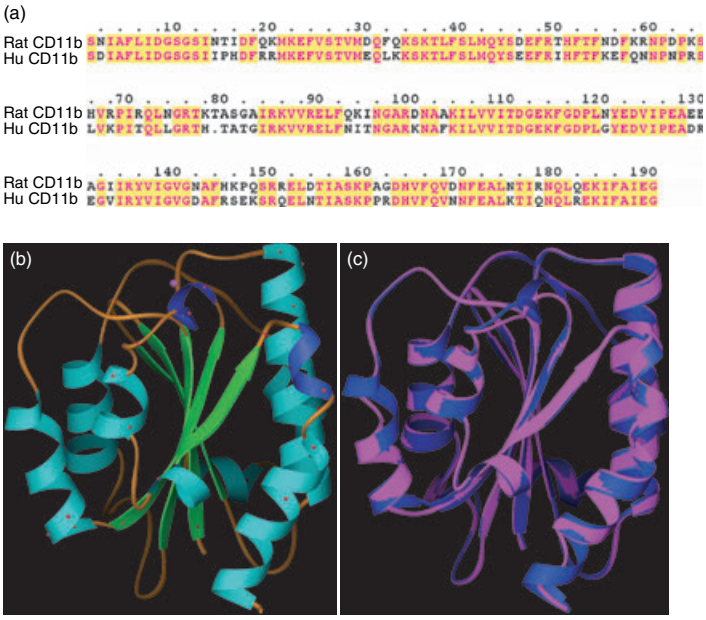

Figure 2. Sequence and structure alignment of rat CD11b A domain. (a) Alignment of the rat and human CD11b A-domain amino acid sequence. The rat CD11b A peptide sequence is ~70% identical to the human CD11b A-domain (a), with a single insertion at Ala212. This residue is located near the N-terminus of the a4 helix, three amino acid residues away from the conserved metal-ion-dependent-adhesion-site (MIDAS) residue Thr209 and should not impact MIDAS coordination by T209. Residues invariant in the two compared sequences are boxed in yellow. (b) Ribbon structure of the rat CD11bA, developed with MODELER 7·7, based on the structure of human CD11bA (PDB id: 1bho).[28] Sphere in magenta shows the putative metal ion in MIDAS. The non-conserved residues in rat CD11bA are distributed throughout the structure (b, red dots in a structure model of rat CD11bA starting from Asn30 to Gly218). (c) Superimposition of rat CD11bA (in magenta) with that from humans (in blue). The structure model of rat CD11bA is superimposable on that of human CD11bA with a root mean square difference of 0·149 Å for 189 Cα atoms.

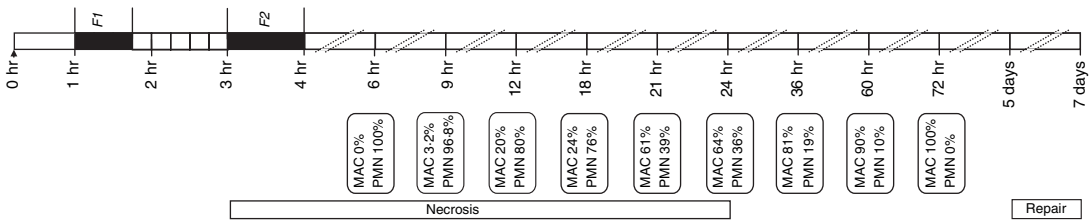

Figure 3. Chronology of the inflammatory lesion in the rat model of skeletal muscle injury model. F1 = first wave of polymorphonuclear cells (PMN), F2 = second wave of PMN. The profile of inflammatory cells at various time-points post-injury is listed. Time scale after 4 hr is not drawn to scale (interrupted lines).

presented in Fig. 3. Histological examination of injured muscle revealed erythrocyte leakage into the injured area, oedema, myofibre disruption and necrosis (Fig. 4). The acute inflammatory response to mechanical trauma was also characterized by an early (within 30 min post-injury) wave of PMN influx into the injured site and the endomesium 5–10 mm away from the immediate site of haematoma formation, followed by a second phase 3 hr post-trauma that lasted up to 24 hr (Fig. 4, panels 2–5). Macrophages were observed around the injured area 9 hr post-injury (Fig. 4, panel 6) and active resorption of the inflammatory lesion was observed 60 hr after injury (Fig. 4, panel 7). After 72 hr, only macrophages were present in association with active wound resorption (Fig. 4, panel 8). At day 7, resorption was almost complete with histological evidence of muscle fibre fibrosis (Fig. 4, panel 9).

Leucocyte influx and muscle fibre protection in mAb- and recombinant CD11bA-treated rats

Histological examination of inflamed muscle of rats that received the anti-CD11b function-blocking mAb OX42[38] showed a significant decrease ($P < 0{\cdot}01$) in the number of infiltrated PMN (86 ± 2%, $n = 50$) (Fig. 5a) and protection of the muscle fibres outside the immediate zone

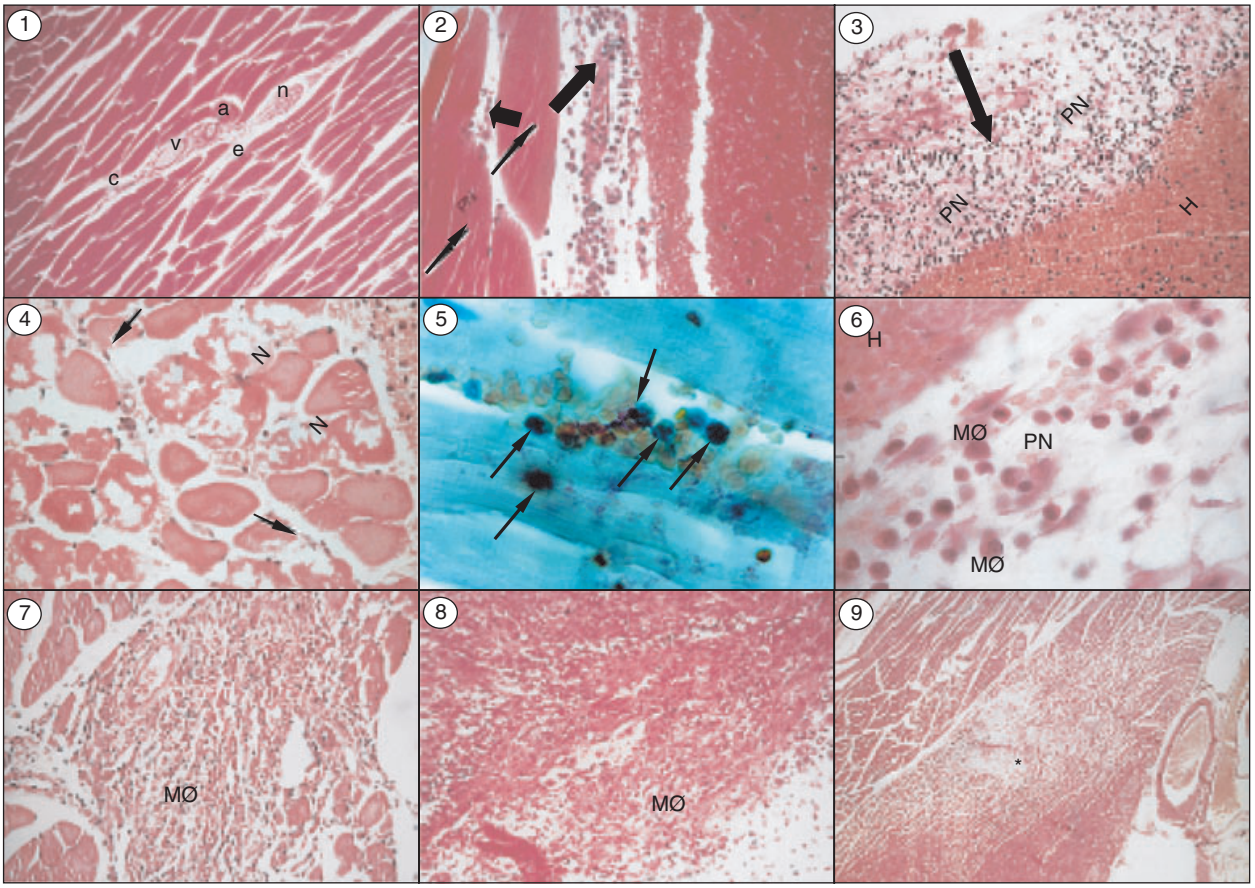

Figure 4. Histology of skeletal muscle injury in the rat model. All photographs are sections of paraffin-embedded skeletal muscle tissue stained with haematoxylin and eosin. Panel 1: control showing normal myofibre structure of the rat skeletal muscle. a = artery, v = venule, c = capillary, n = nerve, e = extracellular matrix (endomesium). Magnification × 100. Panel 2: influx of the polymorphonuclear cells (PMN) (arrows) into the extracellular matrix adjacent to the injured area (hamatoma, H) 2 hr post-injury. Magnification × 200. Panel 3: infux of PMN (arrow) migrating towards the injured area haematoma (H), 02·45 hr post-injury. Magnification: × 200. Panel 4: myofibre necrosis (N) distant from the injured area 3 hr post-injury. This necrosis is associated with the presence of infiltrating PMN. Non-altered myofibres located between the haematoma and the necrotic area are indicated by arrows. Magnification: × 200. Panel 5: myeloperoxidase staining of infiltrating PMN (arrows) present at the inflammatory site as visualized colorimetrically. Panel 6: inflammatory granuloma surrounding the injured area (H) 9 hr post-injury. In addition to PMN (arrowhead), some macrophages (arrow) are also seen. Magnification: × 1000. Panel 7: injured muscle area 60 hr post-injury. An active resorption coincident with the accumulation of macrophages is present. Only few necrotic myofibres are still observed. Magnification × 100. Panel 8: inflammatory granuloma at 72 hr post-injury. Only macrophages are present at this time-point as assessed morphologically. Magnification × 100. Panel 9: image of wound healing at day 7 post-injury: fibrosis (*) of the injured muscle area. Very few cells of inflammatory origin are still present. Magnification × 40.

of necrosis (84 ± 1%, $n = 50$) up to 4 hr after injury. No effect on leucocyte infiltration or muscle fibre protection were seen in rats who received the isotype-matched controls mAb, PBS or GST alone (Fig. 5a). In rats treated with 1 mg of rsCD11bA, a significant ($P < 0{\cdot}01$) block in PMN transmigration was observed (78% +4%, $n = 70$) 4 hr post-injury (Fig. 5a). At this time-point, severe or moderate necrosis outside of the immediately injured muscle area was no longer observed in 75 ± 4% ($n = 70$) and 84 ± 1% ($n = 50$) in the anti-CD11b the treated rats. Similarly, use of 2 mg of rsCD11bA–GST fusion protein inhibited leucocyte transmigration (78 ± 4% ($n = 30$) and prevented tissue necrosis 77% ± 2% ($n = 30$) (Fig. 5a). Representative histological sections reflecting the reduction in leucocyte infiltration (Fig. 5b, panels 2 and 4) and tissue necrosis (Fig. 5b, panel 3) in areas adjacent to the mechanical injury (Fig. 5b, panel 1) 3·5 hr post-injury are shown. Animals that received PBS, GST or one of the three non-functional CD11bA mutants showed a pattern of PMN transmigration and tissue necrosis that are similar quantitatively and qualitatively to those observed in the control groups of non-treated animals at 3·5 hr post-injury. In animals treated with 1 mg ($n = 20$) or 2 mg ($n = 10$) of rsCD11bA–GST fusion protein, PMN infiltration at 8 hr post-injury was equivalent to that observed between 3 and 4 hr in the non-treated

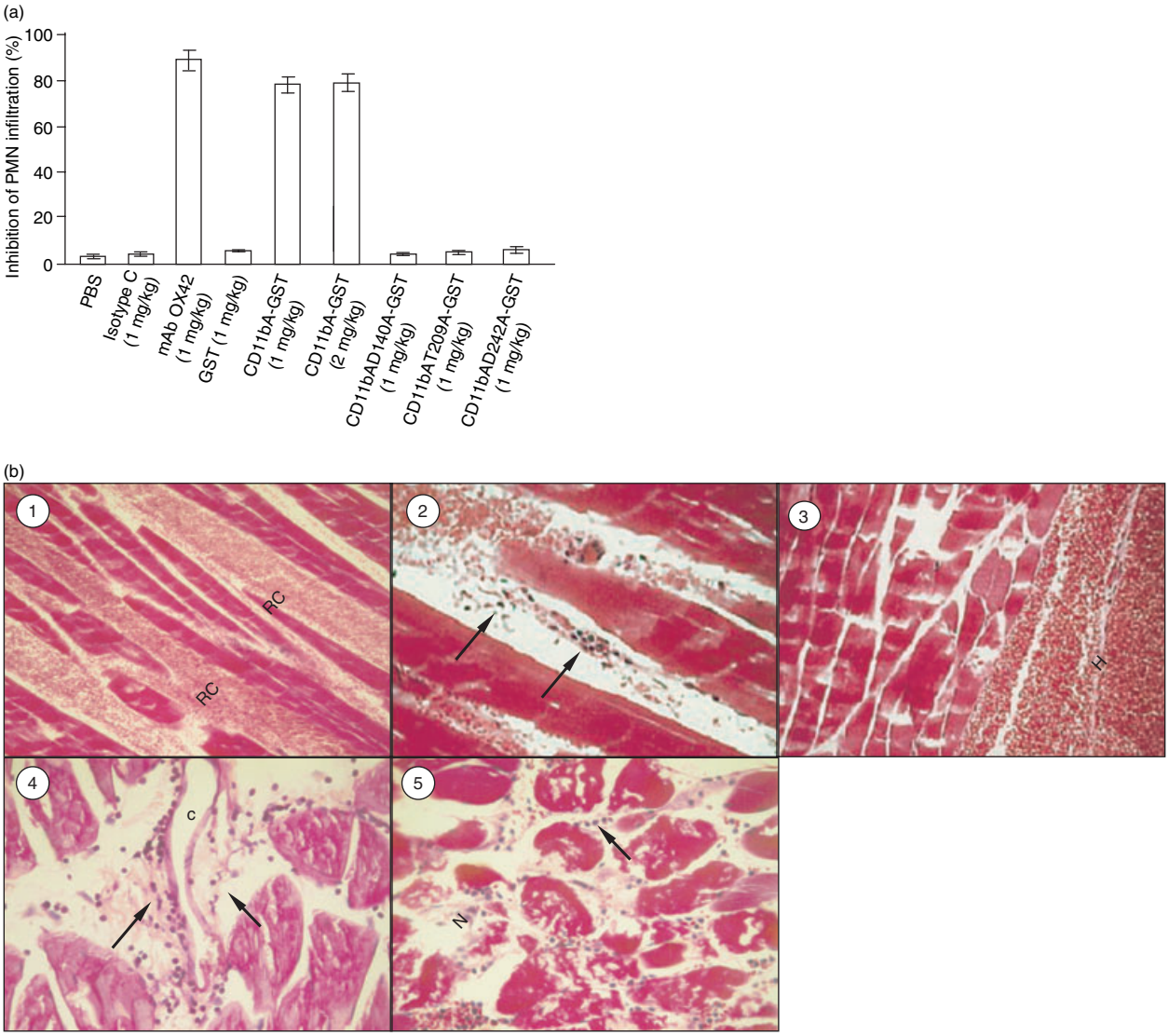

Figure 5. (a) Study of polymorphonuclear cells (PMN) infiltration into inflamed skeletal muscle following administration of an anti-CD11b monoclonal antibody (mAb) (OX42), two concentrations (1 and 2 mg) of wild-type CR3 glutathione-S-transferase (GST)-A domain fusion protein, three different mutated forms of this polypeptide and as control mAb (OX42) isotype, phosphate-buffered saline (PBS) and GST. Significant reduction of the number of infiltrated PMN is observed only with mAb (OX42) and rsCD11bA. (b) Leucocyte influx and muscle injury in rsCD11bA–GST-treated rats. Histological examination of haematoxylin and eosin-stained muscle tissue that was obtained 3·5 hr (panels 1–4) and 7·5 hr (panel 5) following injury. Panel 1: myofibres close to the injured area. Red blood cells (RC) that leaked from ruptured vessels are spread over the tissue. Magnification × 100. Panel 2: myofibres 5 mm away from the injured muscle area is normal 3·5 hr post-injury. The presence of PMN in some congested vessels is observed. Magnification × 100. Panel 3: haematoma with no organized inflammatory granuloma around. Magnification × 100. Panel 4: section of a capillary (c) located in close vicinity of the injured area. Only few leucocytes (arrows) were able to transmigrate. Magnification × 200. Panel 5: extracellular matrix (endomesium) 5 mm away from the injured area 7·5 hr post-injury. Some nectrotic (N) myofibres together with infiltrating leucocytes (arrows) are observed. Magnification × 200.

control group, as was the severity of tissue necrosis near the injured area (5–10 mm away from the haematoma) (Fig. 5b, panel 5).

Discussion

Neutrophil extravasation through the endothelial lining is a crucial event in the development and progression of leucocyte-mediated tissue injury in most inflammatory diseases. It is a multistep process where each event involves a series of adhesion molecules. Some of these steps are amenable to down-modulation that would attenuate the inflammatory response.[39,40] To prevent leucocyte adhesion to the vascular endothelium and subsequent transmigration, we developed a strategy focused on blocking the firm adhesion step of leucocyte to capillary

endothelium that is mediated by the β2 integrin CR3 (CD11b/CD18) by using its major ligand binding A-domain as a receptor decoy. This strategy was tested in a developed rat model of skeletal muscle injury similar to the muscular contusion model reported by Crisco *et al.*[41] However, our model has a reproducible inflammatory pattern of leucocyte infiltration. The latter occurs in two phases occurring 1 hr and 3 hr post-injury, the first probably originating from the marginal vascular pool where PMN predominate.[42] In our study, we investigated the tissue necrosis taking place at a distance from the site of induced mechanical trauma.

Previous *in vitro* studies have shown that the A-domain of the CR3 alpha subunit, CD11b, is necessary and sufficient for mediating the proinflammatory functions of this receptor, and contains the receptor's major ligand binding sites.[31–33] We have cloned rat CD11b A-domain and produced in recombinant active and inactive forms as a GST fusion protein, recognized by the CD11bA-specific function-blocking mAb OX42, used as a positive control in our injury model, indicating proper folding. Functionally, we have shown previously that the wild-type form of this fusion protein binds *in vitro* to a recombinant form of ICAM1, a natural ligand of CR3.[31] The GST–fusion protein was preferred to the CD11bA alone (24 kDa) because the molecular weight of the fusion protein at 45 kDa is probably cleared somewhat less rapidly by the kidney than CD11bA alone.

The recombinant functionally active fusion protein has proved to be effective in blocking PMN transendothelial migration and in preventing PMN-mediated inflammatory injury in the skeletal muscle. The block in PMN extravasation and the consequent tissue protection observed in our study resulted specifically from the introduction of active CD11bA, as GST alone or fusion proteins carrying non-functional CD11bA were ineffective. Furthermore, concentration of the proteins injected into the animals (1 and 2 mg rsCD11bA, and 1 mg for the mAbs and GST) per kilo of weight was not high enough to affect serum protein concentration significantly. The protective effect observed with the rsCD11bA peaked at 3–4 hr post-trauma, and subsided thereafter. This transient effect may be explained by the rapid renal clearance *in vivo*. The kinetics of disappearance of rsCD11bA from plasma will be needed to confirm this. A second explanation is that the form of CD11bA used in this study contained approximately 10% of the open (high-affinity) conformation of this domain.[43] Methods for generating recombinant CD11b A-domain in its high-affinity state have been described.[44] Similarly, the CD11a A-domain was locked successfully in an active conformation and has been shown to antagonize lymphocyte homing *in vivo*.[45]

Our data are the first to show that the recombinant soluble form of the integrin CR3 alpha chain CD11b A-domain can be used to block diapedesis and to act as a protective agent in an inflammatory model of muscular injury. To obtain a long-lasting anti-inflammatory effect it would be necessary to design an A-domain with prolonged circulatory half-life or to use a form that is naturally locked into the high-affinity conformation. The strategy we have developed could be an alternative to antibodies in targeting integrins[46] to block leucocyte influx in inflammatory diseases. It would particularly benefit inflammatory states where the sequence of events leading to neutrophil extravasation is mediated mainly by any of the integrins that have an A-domain.

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